DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

THE DIVISION OF AIDS

NATIONAL INSTITUTE OF ALLERGY & INFECTIOUS DISEASES

NATIONAL INSTITUTES OF HEALTH

and

COLLABORATING INVESTIGATORS

ROCHE AMPLICOR HIV MONITOR™ ASSAY
Quantitative plasma HIV-1 RNA RT/PCR Assay

I. PRINCIPLE

The Amplicor HIV Monitor™ Test is a PCR system for the quantitative measurement of HIV viral RNA in plasma. The plasma is extracted with a Lysis Reagent containing guanidine thiocyanate and Quantitation Standard (QS) RNA. A known amount of QS is introduced into each sample with the Lysis Reagent to permit quantitation of HIV RNA from a comparison of resulting optical densities following amplification and detection. The RNA is precipitated with isopropanol and resuspended in a buffer containing carrier RNA. A 142 base pair sequence in the gag gene of HIV (primers SK431 and SK462) is amplified by reverse transcription (RT) and Polymerase Chain Reaction (PCR) in a single reaction. The primers are biotinylated at the 5' ends to yield biotinylated amplification products, or amplicons. The biotinylated HIV and QS amplicons are detected in separate wells of a microwell plate (MWP) coated with HIV-specific and QS-specific oligonucleotide probes, respectively. To measure the HIV and QS amplicons over a large dynamic range, 5-fold serial dilutions of the amplicons are made in the HIV-specific and QS-specific wells of the MWP. The bound, biotinylated amplicons are quantified with an Avidin-horseradish peroxidase (HRP) conjugate and a colorimetric reaction for HRP. The HIV RNA copy number is then calculated from the known input copy number of the QS RNA, the optical densities (450 nm) of the HIV well and the QS well that fall within a defined range, and the dilution factors associated with the selected wells.

II. SPECIMEN REQUIREMENTS

Plasma collected in an ACD or EDTA tube and removed from the cells within 6 hours of collection is required for the Roche Amplicor HIV Monitor Assay. The plasma must be stored at -20^0C or below until testing. Freeze/thawing should be avoided. Plasma specimens may be frozen and thawed up to 3 times. Heparinized plasma cannot be used.

III. REAGENTS

VQA standards at 0, 1.5×10^4, 1.5×10^5 and 7.5×10^5 HIV RNA copies/mL. Store at -70^0C.

Roche AMPLICOR HIV MONITOR™ TEST kits. Stored in accordance with manufacturer's specifications.

Isopropanol (reagent grade).

Ethanol, 70% (v/v) prepared from absolute or reagent grade stocks using deionized water.

Distilled or deionized water.

IV. Equipment and Supplies

Area 1: Reagent Preparation

Gloves
Laboratory coat
MicroAmp® Reaction Tubes and caps
MicroAmp® tray, retainers and base (for Perkin-Elmer GeneAmp®
PCR System 9600 or System 2400 thermal cycler)
Micropipettes (adjustable volume, 20-20µL) with plugged (aerosol barrier) tips
Repeat pipettor and individually wrapped tips
Plastic resealable bags

Area 2: Specimen Preparation

Gloves
Laboratory coat
Vortex mixer
Microcentrifuge (minimum RCF 15,500 x g, fixed angle rotor preferred)
Pipettors, 200 µL to 1000 µL volume capacity with aerosol barrier tips
2.0 mL polypropylene, screw cap microcentrifuge tubes
Sterile-filtered deionized water
Sterile disposable polystyrene pipettes (5, 10, 25 mL)

Area 3: Amplification and Detection

Gloves
Laboratory coat
Multichannel pipette or PCR AMPLICOR^{TM} Electronic Impact Pipettor
Pipettors, 50 µL to 1000 µL volume capability
Plugged (aerosol barrier) micropipette tips* (200 µL) and unplugged tips (200 µL)
Perkin-Elmer GeneAmp® PCR System 9600 or System 2400 thermal cycler
Microwell plate washer.§
Microwell plate reader†.
Disposable reagent reservoirs
Microwell plate lid
Incubator $37^0C \pm 2^0C$
Graduated vessels

* Pipettes should be accurate within 3% of stated volume. Plugged (aerosol barrier) tips must be used to prevent sample and amplicon cross contamination.

§ Capable of washing 12 x 8 microwell format with 350-450 µL of Wash Solution per well at 30 second time intervals.

† Microwell reader specifications: bandwidth=10±3 nm, absorbance range=0 to 3.00 A_{450}, repeatability 1%, accuracy 3% from 0 to 2.00 A_{450}, drift 0.01 A_{450} per hour.

V. PROCEDURE

This procedure should be performed in three areas of the laboratory as directed in the following instructions. All reagents must be at ambient temperature before use. Use micropipettors with plugged (aerosol barrier) tips.

A. Master Mix Preparation
Performed in Reagent Preparation Area (Area 1)

1. Prepare Working Master Mix by adding 100 µL of Manganese Solution to one tube of HIV Master Mix. Recap the Master Mix tube and mix well by vortexing 3-5 seconds or by inverting the tube 10-15 times. The pink dye indicates that the manganese has been added to the Master Mix. Master Mix and Manganese are provided in single-use vials, sufficient for 12 tests. Unused Working Master Mix must be discarded.

2. Determine the number of MicroAmp® Reaction Tubes required. The recommended batch size is 12 tests, although a total of 24 tests can be done simultaneously. Place Reaction tubes in MicroAmp® tray, lock tubes in position with tube retainer, and place in base. Place 12 tubes for each batch in a single row.

3. Pipette 50 µL of Working Master Mix into each tube using a micropipettor with a plugged tip or repeat pipettor. Check for pink color to confirm that manganese was added to the Master Mix. Discard unused Working Master Mix.

4. Place the MicroAmp® tray in a plastic, resealable bag, and move to the Specimen Preparation Area (Area 2). Store MicroAmp® tray at 2-8^{0}C until specimen preparation is completed. Amplification must begin within 4 hours of preparation of Working Master Mix.

B. Specimen preparation
Performed in Specimen Preparation Area (Area 2)

1. Prepare 70% ethanol. For 12 tests, mix 11.0 mL 95% ethanol and 4.0 mL of deionized water. This solution should be made fresh on each sample processing day.

2. Prepare the Working Lysis Reagent by adding 100 µL of the Quantitation Standard (QS) to the entire vial of Lysis Reagent. Be sure all crystals in the Lysis Reagent are dissolved before adding QS.

3. Label one 2.0 mL screw cap microcentrifuge tube for each sample and control.

4. Thaw plasma specimens at room temperature and vortex 3-5 seconds.

5. Dispense 600 µL Working Lysis Reagent into each tube.

6. Add 200 µL sample or control to each labeled tube containing Working Lysis Reagent. Immediately cap and vortex 3-5 seconds. When Roche Controls are used:

    a. Add 200 µL of each patient specimen to appropriate tube.

    b. For each Negative and Positive Control, add 200 µL HIV Negative Human Plasma to the appropriate tubes. Cap the tubes and vortex for 3-5 seconds.

    c. Add 50 µL HIV-1 Monitor Negative Control, Low Positive Control and High Positive Control to the appropriate tubes. Cap the tubes and vortex.

7. Incubate tubes for 10 minutes at room temperature.

8. Remove cap and add 800 µL 100% isopropanol to each tube. Vortex for 3-5 seconds.

9. Put an orientation mark on each tube and place tubes into the microcentrifuge with the mark facing outward so that after centrifugation, the pellet will align with the orientation mark. Centrifuge samples at 12,500 x g for 15 minutes at room temperature.

10. Beginning with the control tubes, carefully draw off the supernatant without disturbing the pellet (which may not be visible) using a fine tip, disposable transfer pipette. Slide the pipette down the outside of the tube along the side opposite the pellet while drawing off the liquid. Maintain a continuous negative pressure with the pipette as you draw off the liquid. It is important to remove as much liquid as possible without disturbing the pellet.

11. Add 1.0 mL 70% ethanol to each tube, re-cap, and vortex 3-5 seconds.

12. Place tubes into the microcentrifuge with the orientation mark again facing to the outside so that the pellet will align with the orientation mark. Centrifuge samples at 12,500 x g for 5 minutes at room temperature.

13. Beginning with the control tubes, carefully remove the supernatant as before without disturbing the pellet. The pellet should be mostly white and clearly visible at this step. Remove as much of the supernatant as possible. NOTE: Residual ethanol can inhibit the amplification.

14. Add 400 µL Specimen Diluent.

15. Vortex vigorously for 10 seconds to resuspend the extracted RNA. Note that insoluble material often remains.

16. Amplify the processed specimens within 2 hours or store at -20^{0}C or colder. Pipette 50 µL of prepared controls and patient specimens into the appropriate MicroAmp® tubes containing previously pipetted Working Master Mix, using a micropipettor with plugged tips. Cap the tubes. Apply pressure for a tight seal using the MicroAmp® Cap Installing Tool.

17. Move the tray of capped tubes to the Amplification and Detection Area (Area 3).

C. Amplification

Performed in Amplification and Detection Area (Area 3)
Note: Thermal cycler must be turned on at least 30 minutes prior to amplification.

1. Place the MicroAmp sample tray (without base) into the thermal cycler sample block.

2. Program the Perkin-Elmer GeneAmp® PCR System 9600 or System 2400 for the Amplicor HIV Monitor™ Test as follows:

Hold	2 min. 50^{0}C
Hold	30 min. 60^{0}C
4 cycles	10 sec. 95^{0}C, 10 sec. 55^{0}C, 10 sec. 72^{0}C
26 cycles	10 sec. 90^{0}C, 10 sec. 60^{0}C, 10 sec. 72^{0}C
Hold	15 min. 72^{0}C (not to exceed 15 mins.)

In the Cycle programs, the ramp time and allowed set point error should be left at the default settings of 0:00 (which is the maximum rate) and 2^{0}C, respectively. Link the 5 programs together into a Method program.

3. Start the Method program (the program runs approximately one hour and 30 minutes).

4. Remove the sample tray from the thermal cycler at any time during the final Hold program. Do not allow the tubes to remain in the thermal cycler beyond the end of the final Hold program, and do not extend the final Hold program beyond 15 minutes. **Do not bring amplified DNA into Area 1 or Area 2. The amplified controls and specimens should be considered a significant potential source of DNA contamination.**

5. Carefully remove caps to avoid creating aerosols. Immediately pipette 100 µL of Denaturation Solution into each PCR tube using a multichannel pipettor, and mix by pipetting up and down 5 times. (Program 1, AMPLICOR™ Electronic Impact Pipettor; see instructions). The denatured amplification reaction mixtures should be held at room temperature no more than 2 hours before proceeding to detection. If detection will not be performed within 2 hours, re-cap the tubes and store the denatured amplification mixtures at 2-8^{0}C for up to one week.

D. Detection

Performed in Amplification and Detection Area (Area 3)

1. Warm all reagents to room temperature prior to use.

2. Prepare Working Wash Solution as follows. Examine the Wash Concentrate for precipitation and, if necessary, warm at 30^{0}C to redissolve any precipitate. Add 1 volume of 10X-Wash Concentrate to 9 volumes of distilled or deionized water. Mix well. The volume of Working Wash Solution required depends on model of washer being used. Store Working Wash Solution for up to 2 weeks at 4-25^{0}C in a clean, closed plastic container.

3. Allow Amplicor HIV Monitor microwell plate (MWP) to warm to room temperature before removing from the foil pouch. Add 100 µL of Amplicor HIV Monitor Hybridization Buffer to each well (Program 2, AMPLICOR™ Electronic Impact Pipettor; see instructions). Rows A-F of the Amplicor HIV Monitor MWP are coated with the HIV-specific oligonucleotide probe; rows G and H are coated with the QS-specific oligonucleotide probe.

4. Using a 12-channel pipettor with plugged tips, add 25 µL of each denatured PCR reaction mixture to a separate HIV well in row A of the MWP and mix by pipetting up and down 10 times. Make serial 5-fold dilutions in the HIV wells (rows B-F), as follows. Transfer 25 µL of the mixture from row A to row B and mix as before. Continue through row F. Mix row F as before, then remove and discard 25 µL. Discard pipette tips. (Addition of denatured PCR reactions to MWP and the serial dilutions may be done with Program 3 of the AMPLICOR™

Electronic Impact Pipettor. This program transfers 25 µL, mixes by pipetting 60µL 5 times, and aspirates 25 µL; see instructions).

5. Again, using a 12-channel pipettor with plugged tips, add 25 µL of each denatured PCR reaction mixture to a separate QS well in row G of the MWP and mix by pipetting up and down 10 times. Make one 5-fold dilution in the QS wells of row H, as follows. Transfer 25 µL from row G to row H, mix as before, remove 25 µL from row H and discard. (Program 3 of the AMPLICOR™ Electronic Impact Pipettor may be used.)

6. Cover the plate and incubate for 1 hour at $37^0C \pm 2^0C$.

7. Wash plate 5 times with the Working Wash Solution and an automated microwell plate washer. (Washer should fill wells to 400-450 µL volume and have a 30 sec. soak.) Tap plate dry after Wash.

8. Add 100 µL Avidin-HRP conjugate to each well (Program 2, AMPLICOR™ Electronic Impact Pipettor). Cover plate and incubate for 15 minutes at $37^0C \pm 2^0C$.

9. Wash plate as in step 7.

10. Prepare Working Substrate Solution. For each MWP, mix 12 mL Substrate A with 3 mL Substrate B. Protect Working Substrate from direct light.

11. Pipette 100 µL of Working Substrate Solution into each well of the MWP (Program 2, AMPLICOR™ Electronic Impact Pipettor).

12. Allow color to develop for 10 minutes in the dark, at room temperature,

13. Add 100 µL of Stop Reagent to each well (Program 2, AMPLICOR™ Electronic Impact Pipettor).

14. Measure the optical density at 450 nm (single wavelength) within 10 minutes.

E. Calculations:

1. Choose the appropriate HIV well OD values as follows:

   a. The HIV wells in rows A-F represent neat, 1:5, 1:25, 1:125, 1:625, and 1:3125 dilutions of the amplification products, respectively. The absorbance values should decrease with the serial dilutions, with the highest value for each test in row A and the lowest value in row Fa.

b. Choose the well with the lowest OD reading in the range of 0.200 to 2.00 OD units.

c. If any of the following conditions exist, see Unexpected Results Section.

all HIV OD values <0.200
all HIV OD values >2.000
HIV OD values not in sequence (i.e.,, OD values do not decrease from well A to well F)

2. Subtract background from the selected HIV OD values. Background = 0.070 OD units. Then calculate the Total HIV OD by multiplying the resulting OD value by the dilution factor associated with that well.

Total HIV OD = (Selected HIV OD-0.070)X dilution factor

3. Choose the appropriate QS well as follows:

a. The QS wells in rows G and H represent neat and 1:5 dilutions of the amplification products, respectively. The absorbance value in row G should be greater than the value in row H.

b. Choose the well with the lowest OD in the range of 0.300 to 2.00 OD units.

c. If the following conditions exist, see Unexpected Results Section.

both QS OD values <0.300
both QS OD values >2.000
QS OD values not in sequence (i.e.,, OD values do not decrease from well G to well H)

4. Subtract background from the selected QS OD values. Background = 0.070 units. Then calculate the Total QS OD by multiplying the resulting OD by the dilution factor associated with that well.

Total QS OD = (Selected QS OD - 0.070) X dilution factor

5. Calculate HIV-1 RNA copies/mL plasma according to the formula below.

$$\text{HIV RNA copies/mL plasma} = \frac{\text{Total HIV OD}}{\text{Total QS OD}} \times \text{Input QS Copies per PCR Reaction} \times 40$$

Where:
Input QS copies per PCR reaction is the number of QS molecules introduced into each reaction and is specific to each lot of QS RNA. See the Amplicor HIV Monitor™ Test

Data Card for the Input QS Copies per PCR Reaction. Verify that the QS RNA lot number matches the lot number on the Data Card.

40 = Correction factor to convert copies/PCR to copies/mL of plasma..

6. Unexpected Results

a. All HIV OD values <0.200. If all the HIV wells have OD values less than 0.200, but the QS wells have the expected values, use 0.200 as the HIV OD and 1 as the dilution factor, calculate the result as above, and report result as "No HIV RNA detected; less than... " (the calculated value).

b. All HIV OD values >2.000. If all HIV wells have OD values greater than 2.000, then the HIV copy number is above the dynamic range of the assay. Repeat the entire test (including extraction), first diluting plasma 1:50 with HIV Negative Human Plasma. Calculate the HIV result as above, then multiply the final result by 50.

c. HIV OD values out of sequence. If the HIV wells do not follow the general pattern of decreasing OD values from well A to well F, then an error in dilution may have occurred. Repeat the assay on that sample.

1) In reactions containing a very high HIV copy number, the 1:1 and 1:5 wells can become saturated, turning a greenish-brown color before addition of Stop Solution and a brown color after addition of Stop Solution, resulting in lower ODs.

2) Very high OD values (>3) and very low OD values (<0.1) may not follow a pattern of decreasing OD values from well A to well F.

3) A significant deviation from the general rule of decreasing OD values from well A to well F indicates an error.

4) Both QS OD values <0.300. If both QS wells have OD values less than 0.300, then either the processed sample was inhibitory to the amplification or the RNA was not recovered from the sample. Repeat the assay for that sample including extraction.

5) Both QS OD values >2.000. If both QS wells have OD values greater than 2.000, then an error occurred. Repeat the assay for that sample including extraction.

6) QS OD values out of sequence. If the absorbance of well H is greater than the absorbance in well G, then an error occurred. Repeat the assay for that sample including extraction.

VI. QUALITY CONTROL

VQA standards must be run on the first plate of every assay. Control values are not valid if the QS and HIV absorbance values do not meet criteria described in the Calculation section, or the control results are n valid. The negative control should yield HIV OD values of less than 0.2 OD units in all HIV wells. The positive controls should yield final results that can be found on the Data Card, and should be checked for each lot number (when Roche Controls are used). When VQA standards are used, Controls should yield final results of 1.5×10^4, 1.5×10^5 and 7.5×10^5 copies/mL

A quality control log for the thermal cycler must be completed at the end of every run and show no errors.

VII. REPORTING RESULTS

Results are reported as RNA copies /mL, unless they are too low or too high. If all patient OD wells are <0.20, the result is reported as "No HIV-1 RNA detected, less than... (the calculated minimum value, see V, E, 6, a.) Test results greater than 750,000 copies/mL should be reported as "Greater than 750,000 copies /mL". If quantitation is desired, the original plasma must be diluted and repeated (see V. E, 6, b).

VIII. PROCEDURES NOTES

Racks which hold the processed plasma samples, and the MicroAmp® tray, retainer and base should be soaked overnight in 10% bleach after use to remove any DNA that may be present. Working surfaces in all areas should be cleaned with 10% bleach before and after use. To minimize contamination, the Amplicor HIV Monitor™ procedure must be carried out in the three separate areas, and a uni-directional traffic pattern (Reagent Preparation, Specimen Preparation, Amplification, Detection) must be observed. Use dedicated pipettors and consumables in each area.

IX. REFERENCES

Roche AMPLICOR HIV MONITOR™ TEST Procedure Manual